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Strategies to enhance productivity and modify product quality in therapeutic proteins

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The production of commercially valuable biotherapeutic molecules in mammalian systems has expanded significantly in the last thirty years, but growing economic pressures within the industry are driving efforts to reduce costs and enhance process yields. At the upstream stage, two complementary approaches have evolved to increase productivity and maintain consistent product quality, that is either by altering the cell directly or by manipulating its environment. This review focuses on novel approaches to impact productivity and product quality by manipulating the environment through: (a) altering media composition; (b) modulating operating conditions such as pH and temperature; or (c) intensifying process operations by switching from fed-batch to continuous processes.

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Introduction

The biotechnology industry, a key driver for economic development, has undergone rapid growth since the commercialization of the first recombinant DNA product over 35 years ago [1]. Global sales for biotherapeutics exceeded $200 billion in 2016 [2] and are expected to account for a quarter of the projected $1.4 trillion global spending on medicines by 2020 [3]. As the industry matures, companies face increasing economic pressures stemming from expiring patents, competition from biosimilars, tighter regulations, and decreasing returns on research investments for drug development [4]. Thus, there is a growing need within the biopharmaceutical sector to innovate and improve productivity at each stage in the biopharmaceutical process—from increasing protein expression at the upstream stage, to debottlenecking purification trains and identifying innovative and modular solutions for manufacturing using single use technologies.

At the upstream stage, increasing the production of such complex biotherapeutic molecules comes with the associated challenge of maintaining consistent product quality, thereby ensuring the safety and efficacy of the drug product. The different techniques that have been used to enhance productivity and achieve consistent product quality can be broadly classified into two complementary tactics (Figure 1):

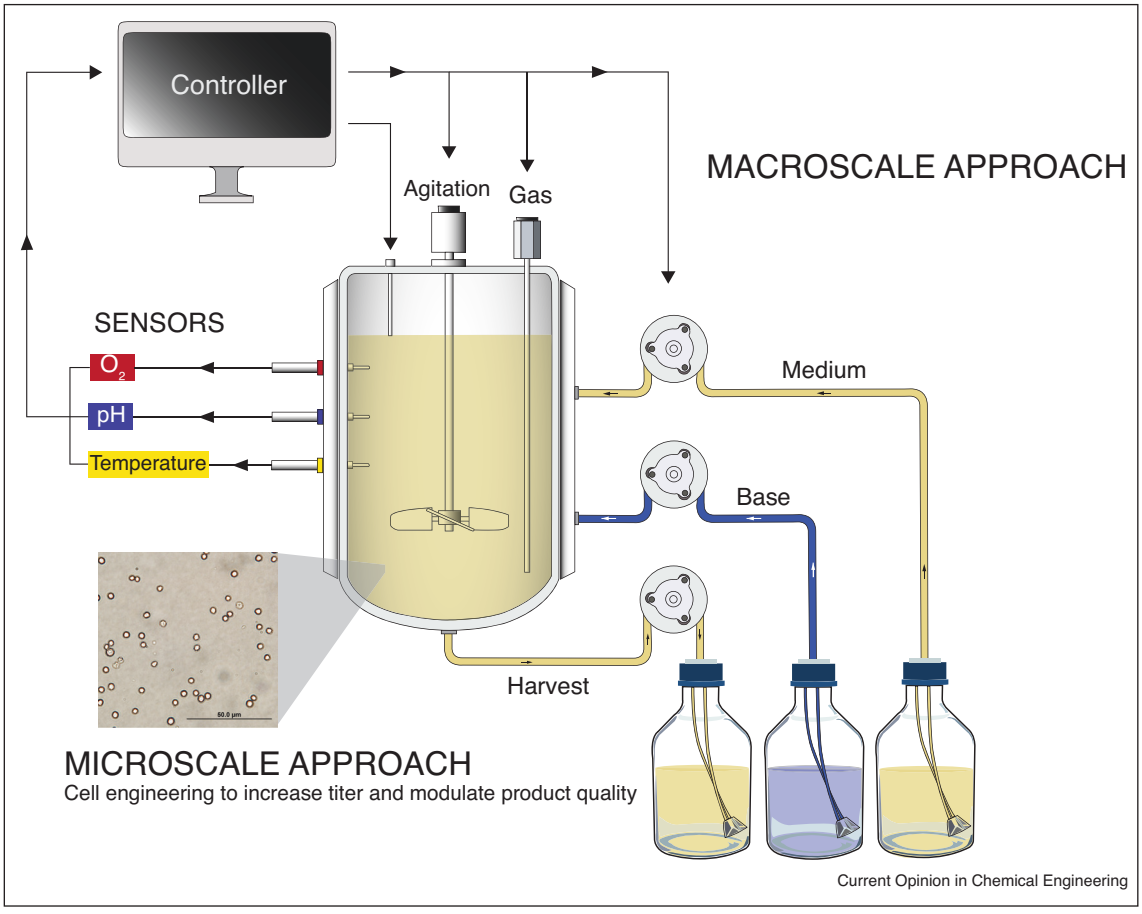
1 Microscale strategies—broadly, these strategies refer to manipulations that are performed at the cellular and subcellular level to identify bottlenecks to protein folding and secretion [5] and/or techniques to alter the final quality profile of the expressed protein [6].
2 Macroscale approaches—when cell line modifications are infeasible, a complementary approach to increase productivity and influence product quality uses factors available at the macroscopic scale by: (a) manipulating media compositions and formulating defined media via media additives and supplements; (b) using optimal process and operating conditions; or (c) altering the mode of operation by switching to perfusion or continuous production.

A detailed discussion of different microscopic approaches using cellular engineering to alter product quality and productivity can be found in other reviews [for e.g. see Refs. [6,7]]. Herein, we limit the scope of the current review to focus on macroscopic approaches to achieve higher titers and consistent product quality by manipulating process and operating conditions.

Media supplements

The advancement of process analytical tools has facilitated a comprehensive analysis of cell culture media and led to the identification of individual components that influence both the productivity and quality of different proteins expressed in mammalian cells (Table 1). For instance, productivity markers were identified in soy hydrolysate using LC–MS/MS and metabolomics, and mechanistic underpinnings for batch-to-batch variations in the process were then established from these insights [8]. Similarly, using NMR and 2D-DIGE, Blondeel *et al.* [9•]

Figure 1



Complementary approaches to modulating productivity and product quality in biomanufacturing. At the molecular level, cell line engineering can be performed to enhance cellular productivity and obtain a consistent or desired quality profile. Alternatively, manipulations at the macroscopic scale, such as changes to the media formulation, shifting process operating conditions, and employing different modes of operation can be utilized to improve productivity and alter product quality for proteins expressed in a specific cell line.

characterized cellular factors limiting growth and productivity and designed rational feeds to enhance productivities and increase cell densities by nearly 75%. By examining different amino acid supplements present in commercially available chemically defined (CD) cell culture media, feed and supplementation strategies have been successfully implemented to increase product titers in fed-batch conditions by minimizing lactate and ammonia accumulation [10,11]. The identification and optimization of media components have also led to strategies wherein media additives have been used to enhance titer. For instance, nucleoside sugars deoxyuridine and thymidine significantly increased the peak viable cell concentration, and consequently antibody titer, when supplemented singly into antibody-producing CHO cultures [12]. Supplementing with deoxyuridine, thymidine, and deoxycytidine together further improved final titers by 17% over cultures treated solely with deoxyuridine [12]. Although these individual approaches are promising and straightforward to test for any protein of interest, the successful implementation can vary in a product and/or process-specific manner, likely in part due to cell line instability and heterogeneity.

A detailed understanding of the effect of different media additives has also resulted in a new set of strategies to modulate product quality attributes. In one instance, researchers observed an increase in tryptophan oxidation in two biopharmaceutical products following a switch from hydrolysate containing media to chemically defined (CD) media [13]. By comparing the individual components of the two media types, they identified and modulated the concentrations of the amino acids, tryptophan and cysteine, and metal ions, copper and manganese to reduce tryptophan oxidation. Vijayasankaran *et al.* [14] have demonstrated that the addition of different media supplements such as hypotaurine, cystine, peptones, and hydrocortisone could be used to reduce coloration and alter the acidic charge variant levels in fed-batch cultures. Similarly, researchers have demonstrated that basic

Table 1

Effect of media and feed supplements on productivity and product quality

Media and feed components added	Titer effects	Growth effects	Glycan effects	Key conclusions
Sugars:				
Raffinose (1–50 mM)	0	0	↑ Mannosylation	High mannose *N*-glycans (M5, M6) [16•,17], downregulated galactosyltransferase, upregulated sialyltransferase, improved antibody ADCC [17] No significant growth/titer changes
'Infrequently used' sugars (1–50 mM)	0	0	↑ Galactosylation	Increases G1F and G2F glycosylation without having major effects on overall culture titer or growth [16•]
Glucosamine (6.5–7.7 mM) Galactose (12.6–15.0 mM) *N*-acetylmannosamine (10.2–10.5 mM)	↑	↑	↑ Sialic acid	Predicted optimum concentrations via statistical modeling. Optimal conditions improved growth and productivity. Increased sialic acid content in albumin-erythropoietin (25.8–33.5%) [19]
1,3,4-*O*-Bu_3ManNAc (200–300 μM)	ND‡	0	↑ Sialic acid	>40% Increase in sialic acid content in erythropoietin; more potent analog of *N*-acetylmannosamine [18]
Deoxyuridine (10–200 mg/L)	↑	↑	ND‡	38–75% increased peak viable cell concentration (VCC) and 37–67% increase in titer between different CHO lines [12]
Thymidine (25 mg/L)	↑	↑	ND‡	28% increased peak VCC and 33% increase in titer; increased peak VCC and titer when combined with deoxyuridine and deoxycytidine [12]
Growth factors:				
LongR3 (IGF-1 analog) (0–200 μg/L)	↑	↑	↑ Sialic acid	Increased sialic acid content, VCC and titer, downregulated cytosolic sialidase Neu2 and decreased extracellular sialidase activity [22•]
Metals:				
Manganese (Mn^{2+}) (0.01–2 μM)	↑	0	↑ Galactosylation	Decreased G0F glycans and concomitant increase in G1F/G2F glycans (up to 20% with just Mn^{2+} and 25% when co-supplemented with Gal) [20•] Time-dependent addition altered glycan distribution. Slight increases in titer and no significant effect on VCC when only Mn^{2+} supplemented at D0 [21]
Zinc (Zn^{2+}) (30–200 μM)	0	↓	↓ Galactosylation	Dose-dependent reduction in cell growth and galactosylation above 100 μM. Mn^{2+} supplementation recovers the galactosylation [20•]
Iron (10–110 μM)	↑	↑	0	VCC increased linearly by 26% from low to high iron. Increase in titer by 37% in highest iron condition and 10% increase in specific productivity. Also increased charge variants in short and long-term culture, coloration, and tryptophan oxidation [15]
Amino acids and derivatives:				
Citrulline (30 μM)	↑	↑	ND‡	Significant increases in cell growth between two mAb-producing cell lines, likely resulting from an increase in intracellular polyamine concentration [8]
Ornithine (90–760 μM)	↑	↑	ND‡	
Proline, serine, cystine, asparagine, glucose, glutamine, hypoxanthine, and choline (17–100% increase relative to concentration in media) Cysteine (1.8 mM) Tyrosine (14.5 mM) Serine (50 mM)	ND‡	↑	ND‡	These 8 metabolites were assembled into a nutrient cocktail which improved cell densities by 75% [9•]
Tryptophan (pre-CD media concentration)	0	0	ND‡	Supplementation with Trp lowered Trp oxidation by 27% [13]
Hypotaurine (13 mM)	0	↑	ND‡	Lowered normalized intrinsic fluorescence by 10–20%, total color by 1.8 units, and acidic charge variants by 8% [14]

0, No change; ↑, increase, ↓, decrease.
ND‡ Indicates factor not discussed.

variants can also be reduced by appropriately modifying cell culture media [15].

One of the most exciting developments in the use of media additives has been in modulating critical product quality attributes such as glycosylation. Supplementation with glucose and galactose are well-known strategies to impact glycan profiles; however, less conventional sugars can also affect glycosylation without significantly impacting culture performance. Hossler *et al.* [16•] reported testing nine 'non-conventional' sugars, consisting of mono-saccharides, di-saccharides, and tri-saccharides, and found eight of them increased G1F and G2F *N*-glycan species, while having nominal or slightly

decreased effects on the final culture growth and titer. The authors believe the observed glycosylation shifts resulted from interactions with glycosyltransferases or cell signaling machinery; this explanation is a departure from conventional nucleotide sugar supplementation like galactose that alters glycan profiles directly as a substrate for galactosyltransferases. Bruhlmann *et al.* [17] observed similar results where another nonconventional sugar, raffinose, was hypothesized to inhibit GlcNAc transferase, resulting in the presence of high mannose glycans on IgG. Chemical precursors and analogs of nucleotide sugars have also recently shown promise for tuning product quality attributes. Betenbaugh *et al.* [18] used 1,3,4-*O*-Bu_3ManNAc, a ManNAc analog, to increase the sialic acid content of erythropoietin (EPO) by 40%. When compared head to head with ManNAc, the analog caused more potent effects at 100-fold lower concentrations and displayed fewer off-target effects. Kim *et al.* [19] also increased sialylation of recombinant albumin-EPO by using a response surface statistical method to identify the optimal concentrations of three supplements, GlcNAc, Gal, and ManNAc.

The addition of metals has been shown to alter antibody galactosylation levels. Zinc chloride ($ZnCl_2$) contrasts the well-known media additive manganese chloride by lowering overall galactosylation in a dose-dependent manner [20•]. Prabhu *et al.* [20•] demonstrated that Mn^{2+} supplementation could reverse and overcome the galactosylation-altering effects of zinc and possibly other trace metal ions present due to variations in media formulation between lots. One potential advantage of using metals as media additives to modify glycan distribution is that they can be altered dynamically by the introduction of chelating agents, or by using a time-dependent media supplementation strategy [21]. Recombinant growth factors can also improve the health and product quality of cells; LongR3 is an insulin-like growth factor that improved sialic acid content and culture viability, and downregulated expression of sialidases in IgG-producing CHO lines [22•]. Integrating both experimental and modeling approaches for optimizing the addition of one of more supplements will also contribute to more finely tuned product quality control.

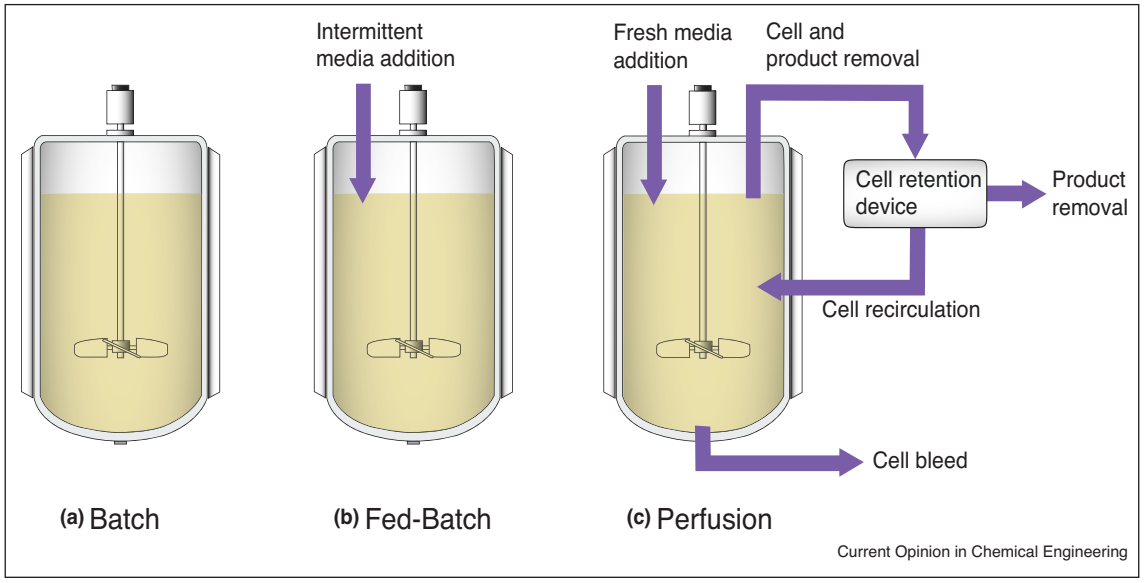
Process operating conditions

Manipulating process operating conditions such as pH and temperature to influence productivity and product quality is a widely used, but not very well understood approach. Hence, researchers are directing their efforts to understand the underlying mechanistic basis for enhanced productivity or altered quality following pH and temperature modulations. For instance, Bedoya-López *et al.* [23] applied next generation sequencing (NGS) to analyze the transcriptomic response in CHO cells under conditions of mild hypothermia and observed differential expression of transcripts that were responsible for avoiding apoptotic cell death and maintaining cellular metabolism, noting that hypothermic conditions extended culture life and increased expression of the protein of interest. Similarly, a direct correlation was established between the upregulation of the transcriptional regulatory factors *myc* and *xbp1s* and hypothermia, resulting in increased viability and productivity during TNFα production [24].

Modulating culture temperature can also have significant impacts on product quality. Temperature shift when coupled with media changes, such as the addition of hydrolysate, can be used to regulate and modify charge heterogeneity. Protein aggregation is also affected by a temperature downshift, as was demonstrated by Paul *et al.* [25] who performed a detailed experimental design to assess the effect of culture conditions on aggregation. Their study noted that in addition to temperature, osmolality, agitation rates, and antifoam addition were critical to control protein aggregation in the bioreactor. Another product quality attribute that is affected by mild hypothermia is glycosylation, as researchers have demonstrated that temperature shift can result in the formation of underprocessed glycan species, by decreasing the availability of intracellular nucleotide sugar donor (NSD) pools [26]. Predictive mathematical models of CHO cell growth, metabolism, antibody synthesis and glycosylation have shown that mild hypothermia results in reduced activity in galactosyltransferase enzyme activity and a change in intracellular NSD pools, resulting in altered glycan distribution profiles [27]. Such a detailed understanding of process conditions can lead to better feeding strategies or cell engineering strategies to achieve comparable glycan distributions under hypothermic conditions.

pH, like temperature, can have a significant impact on cell growth rate, cell density, specific glucose consumption rate and lactate production rate, protein productivity, and quality. This observation has led to efforts to maximize productivity by inducing appropriate pH shifts during different phases of cell growth. Zheng *et al.* [28] observed that antibody production increased, and biological efficacy was significantly enhanced due to changes in glycosylation profiles in pH-shifted perfusion cultures as compared to control cultures. The changes in the glycan profile and subsequent changes in biological efficacy occurred due to the effect of culture pH on the glycosyltransferase enzyme activity. In addition to such experimental observations, model-based approaches have also been developed to fine tune the pH-shift schedule and increase productivity [29]. Further, there is evidence indicating that process conditions in the seed train can have an influence on protein productivity at the production scale. Tung *et al.* [30•] demonstrated that low seed train pH for a specific antibody-producing cell line correlated with an increase in the levels of immunoglobulin

Figure 2



Three different modes of operation in biomanufacturing are **(a)** batch—where cells and media are added at the start and the harvest is collected at the end of the batch run; **(b)** fed-batch—where cells and media are added to the bioreactor at the beginning of the run, with intermittent media additions at specific schedules; and **(c)** perfusion—where there is a continuous feed addition and cell and harvest removal, with the cells being captured in the cell retention device and then returned to the bioreactor.

binding protein (BiP), indicating an increase in cellular stress and activation of the unfolded protein response (UPR) in the cells. Although their findings indicated that the pH-triggered increase in BiP levels was specific to their antibody-producing cell line, it is worth noting that macroscopic factors in the seed train can have significant effects on overall protein yields during subsequent production runs.

Such attempts to generate a mechanistic understanding of the cellular and subcellular changes accompanying process changes will lead to more effective implementation of conventionally used strategies such as temperature and pH shifts for improving productivities.

Mode of operation

Fed-batch processes for manufacturing therapeutic proteins are very well established and have been popular due to their ease of operation, scalability, and process robustness. However, an inherent limitation with fed-batch cultures is that nutrient depletion and by-product accumulation towards the end of the culture may inhibit cell growth or limit productivity. Hence there is growing interest in evaluating continuous process operations such as perfusion, where constant addition of fresh media can be used to replenish nutrients and dilute inhibitory by-products, while continuous harvest and cell recirculation ensures that the product is harvested as soon as it is formed. Figure 2 depicts the differences between batch, fed-batch, and perfusion modes of operation.

Traditionally perfusion processes have been employed for products that are labile or are unstable in batch and fed-batch cultures due to the longer residence times associated with these processes. Researchers have now begun evaluating perfusion systems for production of conventional therapeutic products either in lieu of or in conjunction with fed-batch systems. For instance, Yang *et al.* [31] showed that implementing perfusion in seed train operations followed by fed-batch production cultures resulted in an increase in volumetric productivity for three different CHO cell lines. Hiller *et al.* [32] evaluated a hybrid fed-batch and perfusion process wherein five different mAb producing CHO cell lines were grown under perfusion conditions for four days, and then switched over to fed-batch operations. Using a variation of a previously established technique, they developed a control strategy to activate media feed pumps and harvest removal pumps when the bulk pH in the bioreactor increased following the consumption of lactic acid under glucose limiting conditions. The bioreactors operated using this hybrid perfusion/fed-batch process demonstrated significantly higher titers and improved productivity as compared to the optimized fed-batch process, without altering either the product quality or the run duration. Other researchers have also reported the benefits of using a perfused fed-batch or 'concentrated fed-batch' process using an alternating tangential filter (ATF) and an ultrafilter (UF) to retain both cells and the protein of interest. Using this approach, significant improvements in titers and productivity in different cell lines were

achieved with a marginal change in certain product quality attributes without altering volumetric capacity [33].

With a view to demonstrating the feasibility of continuous manufacturing units for the production of antibodies, Karst *et al.* [34] developed an integrated perfusion and continuous chromatography system wherein they were able to control and fine-tune product quality for the antibody being produced. Additionally, media supplementation strategies and predictive, mechanistic models to modulate the glycan distribution profile in the antibody were implemented in follow-up studies [35]. Other studies have examined the role of perfusion in altering productivity and changing the glycosylation profile. For example, when modes of operation were compared for an anti-CD52 mAb producing CHO cell line, not only did perfusion culture increase productivity, but the antibody also had higher galactosylation and sialylation levels compared to the product obtained from fed batch process [36].

Steady-state operation has also been shown to have an impact on charge variants with one study demonstrating that growth in a perfusion bioreactor resulted in more abundant neutral species and a decrease in acidic and basic charge variants compared to the product from fed-batch cultures [37•]. Interestingly, the authors noted that both fed batch and perfusion cultures had relatively similar glycan distributions indicating the role of media supplements on influencing product quality even in perfusion systems.

Novel perfusion media development efforts from existing fed-batch media have focused on using appropriately balanced concentrations of media components, such as vitamins, lipids, and amino acids, and on the systematic elimination of redundant components [38]. It is important to note that a thorough understanding of cell metabolism can help redirect efforts towards media development and enhance overall productivity while limiting growth when using perfusion media. Further, the use of advanced metabolomic tools should give a greater understanding of the cellular changes under different operation modes, providing tools for more efficient perfusion process design [39,40].

Despite the advantages of perfusion culture for enhancing productivity, widespread implementation and commercial usage has been limited for a variety of reasons. Perfusion systems are operationally complex and require additional unit operations such as cell retention and harvest clarification to be performed on a near-continuous basis. The associated cost of media increases depending on the perfusion rate in the bioreactor; the economic feasibility of perfusion operations thus has been the subject of much research in recent years. Pollock *et al.* [41] have compared different perfusion and fed-batch scenarios for a typical mAb process from pre-clinical to commercial scale for small, medium, and large companies and demonstrated that continuous strategies provide economic and environmental benefits, and have lower risk at product development stages, but not at commercial scales. Similar simulation-based studies by Bunnak *et al.* [42] evaluated the environmental cost associated with perfusion processes based on a life-cycle assessment, where they demonstrated that the inefficiencies in perfusion processes do not stem from bioreactor operations solely, but arise in large part from the downstream processes associated with perfusion. Further, continuous processes could be made as environmentally efficient as fed-batch processes by altering pooling durations. However, such analyses are product and process-dependent, as was demonstrated in a recent publication by Arnold *et al.* [43] who achieved an overall reduction of 15% to their cost of manufactured goods by performing specific modifications to their continuous processing operations, achieving nearly a 4.5-fold increase in volumetric productivity compared to their conventional 500 L fed-batch operation.

With widespread implementation of single use and disposable equipment, advancements in media development, and improvements in cell harvest and protein capture, perfusion processes are likely to become more appealing in the biopharmaceutical industry in the future.

Conclusion

As the biopharmaceutical industry matures into an established industry with ever higher titers being achieved at commercial scales, an evolving concern among practitioners is: what innovative strategies can be implemented to yield maximal benefit for new and established processes? Some researchers have expressed concern that in the case of antibody production, novel technologies might not yield the desired return on investment [44•]. In some cases, applications to novel therapeutics such as antibody-drug conjugates and other proteins have added complexity, in part due to the lower yields and lack of a standard process platform.

There continues to be a concerted effort to achieve the dual objective of high productivity and consistent product quality for all therapeutic products. In this review, we have focused on different macroscale strategies that have been implemented to achieve this objective, that is by (a) modulating media formulation or using medium additives, (b) manipulating operating conditions, or (c) altering the mode of operation from fed-batch to continuous. Advances in analytical technologies, development of inline and online sensors, and increased process robustness and control have resulted in an increasing adoption of these macroscale strategies.

While evaluating each of these strategies, we recognize that their success is incumbent upon the choice of the

appropriate cell line. As demonstrated in a recent study [45] switching media or changing the mode of operation did not alter the innate preferences that different CHO cell lines displayed for protein or biomass synthesis. At the outset of this review, macroscale and microscale strategies were defined as complementary strategies and we note that exploiting the true potential of macroscale strategies requires not only a fundamental understanding of the interplay between the variables at the bioreactor and cellular scale, but also an in-depth understanding of the cell line-specific behavior. A holistic approach that encompasses both microscale and macroscale variables will result in the design of a robust and effective strategy to enhance productivity without compromising quality.

Conflict of interest statement

Nothing declared.

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